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VARIATIONS IN BOTRYTIS CINEREA, PERS., INDUCED BY THE ACTION OF HIGH TEMPERATURES.

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Variations in Botrytis cinerea, Pers., Induced by the Action of High Temperatures.

BY

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With Plate XXXVII and five Figures in the Text.

THE production of variants of *Eurotium herbariorum* from conidia which had been exposed to heat (Barnes (2)) led to experiments with *Botrytis cinerea*. These experiments yielded forms clearly different from the stock strain of *Botrytis*, and the work was continued in order to determine the degree of constancy possessed by these variants.

The strain of *Botrytis* used was obtained in December, 1926, from a strong growth on dead stems of *Helianthus annuus*; cultures were prepared from single conidia, and one of these pure cultures formed the starting-point of the work to be described.

Cultural Details.

The original isolations were made on potato agar; the fungus grew freely on this medium, and formed conidia in abundance, but the crops of sclerotia were poor. Several synthetic media were tried, and of these Czapek's medium,[1] and a modified form of Reidemeister's medium[2] (Reidemeister (13)) were found to give good growth and moderate crops of sclerotia; the second medium has been chiefly used in this investigation.

All the synthetic media were prepared from the same lot of chemicals. Material was sterilized for twenty minutes at thirty pounds pressure; the depth of the medium was maintained at 3–4 mm.

With the exception of those cultures which were incubated for a special reason (p. 829) the cultures have been grown in the open laboratory,

[1] Sodium nitrate, 2·0 grm.: dipotassium phosphate, 1·0 grm.: magnesium sulphate, 0·5 grm.: potassium chloride, 0·5 grm.: ferrous sulphate, 0·01 grm.: cane sugar, 30·0 grm.: agar, 20·0 grm.: distilled water, 1 litre.

[2] Potassium nitrate, 5·0 grm.: magnesium sulphate, 2·0 grm.: dipotassium phosphate, 2·0 grm.: glucose, 50·0 grm.: agar, 20·0 grm.: distilled water, 1 litre.

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and so subject to changes of temperature. During the colder part of the year the temperature ranges between 14–17° C.; in the warmer months it varies with the weather. Cultures were seldom exposed to direct sunlight, but they received much illumination from electric light, amounting probably to ten to twelve hours per day for most of the year. Owing to limitations of space it has been necessary to keep the dishes in stacks of five or six; as far as possible, any effects of uneven illumination due to stacking have been neutralized by frequent rearrangements. It does not seem likely that any given dish has been exposed to conditions differing essentially from those affecting all the cultures in existence at the time.

The Behaviour of the Normal Strain in Culture.

(a) *On Synthetic Media in the Laboratory.*

When cultures are started from conidia, whether transferred by a cool wire or in drops of sterile water, growth is usually just visible to the naked eye in twenty-four hours, and an agar-surface 9 cm. in diameter is covered in about five days. During the first days of growth the mycelium spreads evenly (Pl. XXXVII, Fig. 10) over the medium, penetrates the agar but slightly, and appears as a low, even turf of white and sterile mycelium, 2–3 mm. in height. The aerial mycelium is loose and freely branched; it usually shows but vague indications of radiating main hyphae. By the fourth or fifth day the central region is occupied by a rather dense cushion, about 5 mm. high and 3–5 cm. in diameter, falling steeply to the prostrate margin of hyphae advancing over the medium; immature conidiophores may now be seen on the central cushion. When the margin of the agar is reached appressoria form freely against the glass, and a peripheral ring of aerial mycelium with conidiophores develops rapidly. At this stage conidiophores are forming centrifugally on the central cushion, centripetally on the marginal ring. There was no evidence that the first conidia to mature fall, germinate, and contribute to the development of the colony (Brierley, 6, p. 140); by the time that mature conidia are present it appears that the surface of the agar is too well covered to allow of the establishment of younger colonies.

As the two areas on which conidia are forming approach one another sporulation decreases, so that a somewhat thin crop of conidiophores is formed in a zone lying a centimetre or so from the periphery of the agar; rarely such a zone may be left almost bare of conidiophores.

The conidiophores branch freely. Owing to the dense growth in cultures of the stock strain it is impossible to follow the branching, but in the thinner growths of some of the variants, and of the stock strain on poor media, it can be seen that the first fertile axis bears a terminal head

of spores, with a whorl of branches arising immediately below it; each branch repeats the development, and so on. In this way the dense turf of conidiophores is built up.

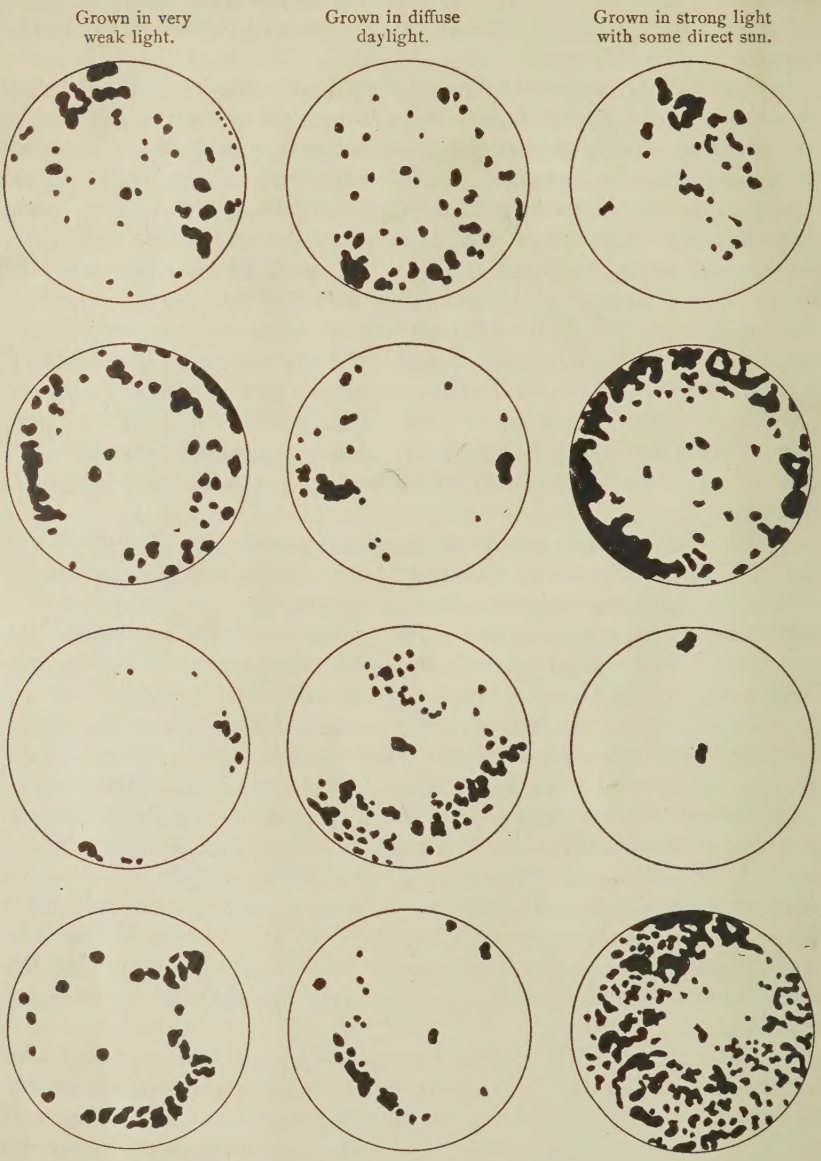
A culture ten days old, viewed from above (Pl. XXXVII, Fig. 1), appears to consist of an even turf of brown conidiophores, but the presence of the thin zone is revealed when the culture is held against the light. Cultures of this age show the first signs of sclerotia as small, whitish hyphal aggregations lying on the medium where conidia are few. The rudiments soon enlarge, remain white for a day or two, then become yellow and translucent, and finally black and opaque: the edge darkens first, and the larger the sclerotium the slower the darkening of the centre. After the first rudiments have appeared, others form nearer the periphery, some against the glass; finally, a few belated sclerotia may arise in the central region. Rarely, these fail to mature, and remain as thin brownish aggregations of indefinite outline; if they blacken they may do so more slowly than those already formed. In cultures three weeks to a month old the sclerotia are usually all black and opaque, and no more develop.

Individual sclerotia attain an average diameter of 3 mm. They are flat, a millimetre or so in thickness, or, if convex upwards, present an illusory appearance of thickness, for the convexity is due to the upward curvature of the sclerotium, with some inclusion of the medium in the lower face. The crusts formed by the fusion of several rudiments usually remain flat, and seldom exceed 1 cm. in any horizontal dimension.

The richness of the crop of sclerotia may be influenced by the manner in which the culture is started. In single spore cultures, or in cultures started from a few conidia placed in the centre of the dish, or from two or three conidia well spaced on the surface of the medium, a crop of seventy to over a hundred sclerotia may be obtained; from dense sowings sclerotia are seldom obtained. However, cultures have been observed in which sclerotia have been scantily produced, or have failed to appear, even when all conditions would seem to have favoured their development. The tracings given in Text-fig. 1 show how erratic the production of sclerotia may be in cultures prepared in the same way and subjected to the same treatment.

When cultures are started from sclerotia they develop much like those from conidia. The production of aerial mycelium and of conidiophores is, however, greatly reduced, and the crop of sclerotia is increased, several hundred often being obtained; conidial transfers from such cultures show the ordinary behaviour.

As cultures age further developments are noted. When there are few or no sclerotia, and the culture is about a month old, tufts of aerial mycelium arise among the old conidiophores, from hyphae on and in the medium.



TEXT-FIG. 1. Twelve cultures of the normal form of *Botrytis cinerea* grown on Reidemeister's medium. All were inoculated with a few conidia placed in the middle of the agar. The erratic production of sclerotia is clearly shown. Camera lucida tracings. $\times \frac{7}{18}$

These tufts may be loose, and greyish or whitish; less often they are dense, and then distinctly tinged with pink, or, if developed in strong light, greenish. The looser tufts form large, pitchy black appressoria against the under side of the lid; the denser tufts usually form smaller appressoria, which may remain brown and translucent. Very few conidiophores are formed on these tufts.

When sclerotia are present, and are five to six weeks old, they give rise to dense groups of conidiophores which do not branch freely; after the conidia are ripe, tufts of whitish, and usually sterile mycelium, arise from and around the sclerotia.

The formation of such tufts of aerial mycelium is a general phenomenon in old cultures. As a rule they remain localized, and attain a diameter of 1–2 cm., but they may increase so much as to occupy completely all the free space in the dish, hiding the older growth; ultimately all collapses to a thin, dirty brown, papery sheet lying on the medium. The production of the tufts is usually accompanied by the secretion into the medium of a brownish-purple or blackish stain, lying below the points of origin of the renewed growths. Cultures inoculated with pieces of mycelium, or with conidia, taken from the tufts, do not show any abnormality.

Appressoria are formed freely by the stock strain. They appear as soon as the spreading mycelium touches the side of the dish, and develop readily against pieces of glass strewn on the surface of the agar. At first white and translucent, they soon turn brown, and then blacken; they may attain a diameter of over 1 mm. and simulate small sclerotia. Small appressoria are also formed in large numbers by the peripheral ring of aerial mycelium, and lie in a crowded thin circular line where the lid rests on the side of the dish.

The foregoing account is based on the observation of the stock and control cultures which have been made during the investigation; of these cultures, 239 in number, none has shown any marked divergence in its characters. Observation of these cultures has not suggested that the stock strain has shown any indication to vary during nearly three years of culture in the open laboratory; nothing has been seen in any of the cultures which could have been isolated and grown as a recognizable variant. Further, it has not been possible to show that transfer from one medium to another may lead to the development of any change in the behaviour of the fungus, other than an obvious and temporary reaction to the medium.

(b) *Incubated Cultures of the Stock Strain.*

Systematic work on the effect of incubation at various temperatures on the stock strain was not undertaken; from time to time, however, some

cultures were grown in incubators, in order to shed light on some of the peculiarities of the variants.

At temperatures between 25° C. and 27° C., on Reidemeister's medium, the general rate of growth did not appear to be essentially different from that observed in control cultures in the laboratory at about 20° C., but the production of conidia was less, and that of sclerotia slightly increased. In one culture, sclerotia formed after six days, and blackened normally. The production of sterile aerial mycelium was increased, and this, as well as the appressoria, often had a pinkish tinge. Greenish tufts also appeared, and in these, as well as on the surface mycelium, microconidia were found. In two cultures grown at 27° C. microconidia were so profusely developed on the surface of the agar that wide, dark green areas appeared, similating infection by *Penicillium*. In incubated cultures microconidia were obtained in five days ; in cultures at laboratory temperatures they have rarely been seen, and then only after a month or so of growth.

More interesting results were obtained at slightly higher temperatures. Growth did not occur at a temperature higher than 31° C. On Reidemeister's medium, at 30° C., growth was very poor. It usually stopped after incubation for eight days, when the colonies had attained an average diameter of 15 mm.; controls of the same age have a diameter of 90–92 mm. The growth was low, completely sterile, and consisted of a mass of irregular swollen, darkly-stained hyphae, with vacuolate contents, covered by a short turf of thin whitish filaments. Beneath the centre of the colony the medium was stained black, and in general appearance the cultures strongly resembled cultures of *Cladosporium*. Some of these cultures were left in the incubator for a further seventeen days, without any change in size or aspect. Transfers of mycelium failed to produce growth, either in incubated cultures, or at ordinary temperatures. Yet, when the cultures were brought back to laboratory temperatures, new growth soon appeared, and in a few days a perfectly ordinary growth, with conidiophores and sclerotia, surrounded the dense colony, above which growth did not appear.

Although sporulation was diminished or suppressed by growing the stock strain at rather high temperatures, and although definite changes in the manner of growth were obtained in this way, transfers of conidia formed in these cultures, either in the incubator or after return to a lower temperature, always gave normal cultures at laboratory temperature.

Efforts were also made to produce the white sterile form described by Beauverie (3, 4) and by Beauverie and Guilliermond (5). Wet earth in Petri dishes was inoculated with conidia of *Botrytis*, and repeated transfers were made of mycelium obtained in the incubator and of conidia obtained after the cultures had been placed in the laboratory. Growth was always very weak, but the white sterile form did not appear. Similar attempts

were made with Petri dishes incubated inside a closed vessel containing water, to maintain a humid atmosphere, and with stoppered bottles containing wet earth. The white form did not develop, and some conidia always appeared when the cultures were allowed to stand at laboratory temperatures. Cultures started from these conidia showed nothing abnormal. Brierley (6) records that he could not obtain the sterile form.

These experiments with incubated cultures were not carried far, but enough was done to show that the strain of *Botrytis* used is extremely sensitive to rise of temperature, and shows a rapid falling off in the rate of growth even when the rise is slight. The production in incubated cultures of much sterile aerial mycelium at the expense of sporulation, the early development of microconidia, the appearance of a pink colour in the aerial mycelium and appressoria, and, at higher temperatures, slackening of the rate of growth and staining of the medium, are all characters which have been found in one or other of the variants presently to be described.

(c) *The Effect of Hot Weather on the Stock Strain.*

The hot weather in July, August, and September, 1929, furnished indications supporting the observations made on incubated cultures. During this period the laboratory temperature remained for days not lower than 20° C., and sometimes rose above 30° C. The cultures of the normal form developed much aerial mycelium, showed great reduction in the crops of conidia, formed many pink appressoria, frequently stained the medium, and yielded few sclerotia. In subsequent cooler weather these abnormalities disappeared, and the strain resumed its ordinary behaviour.

Experiments with Heated Spores.

During 1927, after it had become apparent that the stock strain was reasonably constant in character, a few attempts were made to obtain growth from heated spores; the methods used were those employed at the time in the work on *Eurotium*. It was found that the conidia of *Botrytis* were more readily killed by heat than were those of *Eurotium*, that there was delay in the germination of some of the surviving spores, that a tendency to the production of an enhanced crop of aerial mycelium was apparent, and that some of the sclerotia darkened more slowly than normal ones, requiring three weeks to a month for the completion of the process The aerial mycelium was usually sterile, but occasionally bore a few weak conidiophores which seldom yielded good conidia. It was often greenish in tinge, but though microconidia were suspected they were seldom seen; most of the green colour was due to pigmentation of the hyphal walls.

In 1928, when the experimental work on *Eurotium* was completed, further attempts were made to obtain changes in *Botrytis*. For most of

the trials the following method was used. Weak suspensions of conidia were made in distilled water and shaken until it appeared that the spores were well distributed in the liquid. Quantities of 0·5 cc., were withdrawn by means of a narrow tube, and conveyed separately to the bottoms of test tubes, care being taken to avoid the deposit of liquid on the sides of the tube. The charged tubes were immersed as deeply as possible in a water bath at a known temperature, and held there for two minutes, with persistent shaking, so that the small volume of suspension was thrown in a thin layer against the hot sides of the tube. After withdrawal from the bath, the tube was cooled under a bell-jar; later, drops of suspension were withdrawn on a glass rod, and conveyed to plates of agar. Control cultures were inoculated from the unheated suspension. Suspensions of constant density could not be prepared, but in most of the suspensions used, an average drop, such as was used for inoculating a plate, contained about thirty spores.

All water and apparatus were sterilized before use, and all necessary precautions were taken to avoid contamination. It was found convenient to use test-tubes closed by means of a specimen tube of slightly greater diameter inverted over the mouth; this arrangement formed a sort of deep and narrow Petri dish, and manipulation was easier than when a plugged tube was employed.

In all, 398 attempts were made in this way, including 42 controls. The exposures to heat were made over the range 48–80° C., at intervals of 2° C. The controls all gave normal cultures. Of the 356 dishes inoculated with heated spores only five gave any growth. Four had received drops of suspension which had been heated to 54° C.; the suspension in the fifth had been heated to 66° C. Of the first four, one culture was apparently quite normal, two were distinguished by the production of much aerial mycelium, and one yielded colonies of rather loose growth; these four cultures were not further investigated. The fifth culture ultimately gave some light coloured sclerotia; it was the starting-point of the subsequent work, and is described as culture A, on p. 834.

The absence of contamination from any of the dishes of this long series is an indication of the trustworthiness of the methods used.

A few cultures were started from singed spores; a mass of hyphae and conidia was taken on a hooked wire, well singed in a flame, and transferred to a dish. Growth seldom arose from the charred mass, but conidia which had fallen clear of it during transfer frequently germinated. Colonies from these generally produced a tuft of sterile aerial mycelium when they encountered the inoculum, suggesting the presence in it of a substance capable of affecting the growth of the fungus and causing sterility. As viable spores could be shaken out of the transferred material, it seems likely that similar spores remained inside it. Their usual failure to germi-

nate was probably due to the presence of deleterious substances in the charred mass. The more striking abnormalities observed in these cultures occured in mycelia starting clear of the inoculum, and sometimes not reaching it; the changes noted may therefore be reasonably ascribed to the direct effect of heating upon the spores.

A dense, black stained growth appeared as a single colony in two cultures inoculated with singed spores; in a third it was accompanied by one normal colony. In all three growth arose from spores which had fallen clear of the charred material. The mycelium consisted of a mass of blackish hyphae with barrel-shaped segments, lying on and in the medium, covered by a scanty tuft of unhealthy-looking white hyphae. Although attempts at the formation of conidiophores were seen, conidia could not be found; efforts to obtain subcultures from the colonies, by transfer of mycelium, were fruitless. In its general characters this form resembled the stunted growths obtained when the ordinary form is grown on Reidemeister's medium at 30° C., but, unlike those growths, it did not yield conidiophores at laboratory temperatures.

A low, densely branching colony, also grown from a singed spore, appeared to be identical with the dense white form which will be described in Series I, VI, and VII; it was the only colony to develop in the dish.

Many cultures derived from heated spores were distinguished by the production of an unusually heavy crop of aerial mycelium, with reduced formation of conidiophores and occasional development of faintly green tufts of hyphae. All these phenomena were encountered in cultures of the normal form, grown in the incubator; some were also observed in old control cultures. Their appearance in young cultures, inoculated with heated spores, indicates a slight disturbance of the normal course of development. Efforts were not made to perpetuate these slight changes, nor were attempts made to obtain subcultures from the slightly modified forms numbered 7 and 8 in Table I. All were probably temporary modifications, and would not have repaid investigation.

As in the work on *Eurotium*, so with *Botrytis*, the most striking variants were observed in cultures in which few or only one spore germinated; when a number of spores germinated after exposure to heat, the cultures gave either normal growth, or slightly altered forms, often only recognizable as variants in early stages of growth.

The experiments with heated spores were discontinued as soon as a fair number of positive results had been recorded. The investigation was regarded as an extension of the investigation of *Eurotium*, and it was not considered necessary to accumulate a large number of abnormal forms.

TABLE I.

Summary of Experiments with Heated Spores.

	Method of Heating.				
	Hot Lead.	Hot Wire.	Singed Spores.	Hot. Water.	Totals.
Cultures from heated spores :					
1. No growth	60	2	11	351	424
2. Dense, black stained growth	—	—	3	—	3
3. Low growth with dense branching	—	—	1	—	1
4. Slowly darkening sclerotia	2	1	4	1	8
5. Much aerial mycelium, often with a green tinge, and with greenish appressoria	3	12	19	2	36
6. Greenish aerial mycelium with delayed formation of conidia	—	—	12	—	12
7. Weak growth in young stages	6	7	1	—	14
8. Loosely growing colonies	—	1	—	1	2
9. Normal growth	—	7	12	1	20
Totals	71	30	63	356	520
Controls, all with normal growth	23	8	24	42	97

The more important abnormalities are included under items 2–4 of Table I. The large number of experiments in which all the spores were killed is noteworthy.

THE VARIANT FORMS.

I. THE ORIGINAL VARIANT CULTURES.

Culture A. In April 1928 a single colony appeared in a dish inoculated with a suspension of spores which had been heated to 66° C. for two minutes. Indications of growth were seen five days after inoculation, and it was clear that only one spore had germinated. Once started, the colony grew as fast as ordinary colonies, but at an age when a control culture contained an even growth of old conidiophores, and black opaque sclerotia, Culture A showed much whitish, sterile aerial mycelium and a few whitish sclerotia ; the appressoria were pale yellow. When the culture was 43 days old it contained 21 black opaque sclerotia, all bearing tufts of conidiophores, and eight small, light coloured ones not bearing conidiophores. The culture was kept under observation for a further 62 days ; five light coloured sclerotia were still present. They were removed separately, well rubbed with sterile glass needles in six changes of sterile water, and used to start fresh cultures. Three, each about 1 mm. in diameter, were transferred whole ; two others, each about 2 mm. in diameter, were halved, and used to inoculate four more dishes. All the

sclerotia were discoid and soft ; the two which were cut showed slight superficial darkening and a white medulla.

Growth was obtained in all seven, and in contrast to cultures started from normal sclerotia none of these seven yielded many sclerotia. Such of the seven as formed the starting-points of series of cultures will be described in the series to which they belong.

Culture B. In March 1928 a colony derived from spores heated in a capillary tube on molten lead produced two sclerotia which were still translucent in early July. These sclerotia were washed and transferred separately to fresh media. The resulting colonies produced few conidia, much brownish aerial mycelium, and stained the medium strongly. Few transfers were made from these cultures. A similar variant was afterwards obtained in the progeny of Culture A ; it is described in Series II.

II. The Series of Variant Forms.

Almost all of the cultures of the series now to be described were grown on Reidemeister's medium, and all were grown at laboratory temperatures. When a variant was first recognized, a single colony was isolated as a starting-point for further investigation, or, when conidia were slow to form, the end of a hypha was transferred. Conidia were generally used in making the routine transfers, and these were taken from cultures which showed most strongly the characters of the variant to be perpetuated. From time to time small portions of mycelium were transferred from regions showing the abnormalities characteristic of young cultures of the variants. As it was desired to find how long the variants could preserve their characters under the conditions of culture employed, few subcultures were made from cultures showing a definite approach to the normal form.

The numbering of the series does not denote the order in which they arose. They are described in the most convenient sequence.

Series I.

The original culture of Series I arose from half a sclerotium taken from Culture A. A heavy, white, and sterile cottony mycelium formed, and bore conidiophores freely by the tenth day. Rudiments of sclerotia appeared when the culture was 29 days old, but good sclerotia never developed. Transfers were made from this culture when it was 19 days old ; these cultures resembled the parent in the production of much cottony aerial mycelium. In one, sclerotia never formed ; in the other a few sclerotia appeared, and of these the larger blackened somewhat, but were still translucent after 89 days, while the smaller remained pinkish and light in colour. Conditions of this sort recurred in cultures made during August and September 1928, with indications of the formation of a central mass of indifferently formed sclerotia, recalling the phenomena described more fully in Series III. Not until November 1928 did it become clear that

the cultures of Series I were settling down to two distinct types; since that time these have persisted, though in many of the cultures, as will be shown below, the two have appeared together. In association with the rather weak tendency to produce pink sclerotia noted from time to time in this series, indications of poor growth, similar to the phenomena common in Series III, have been observed.

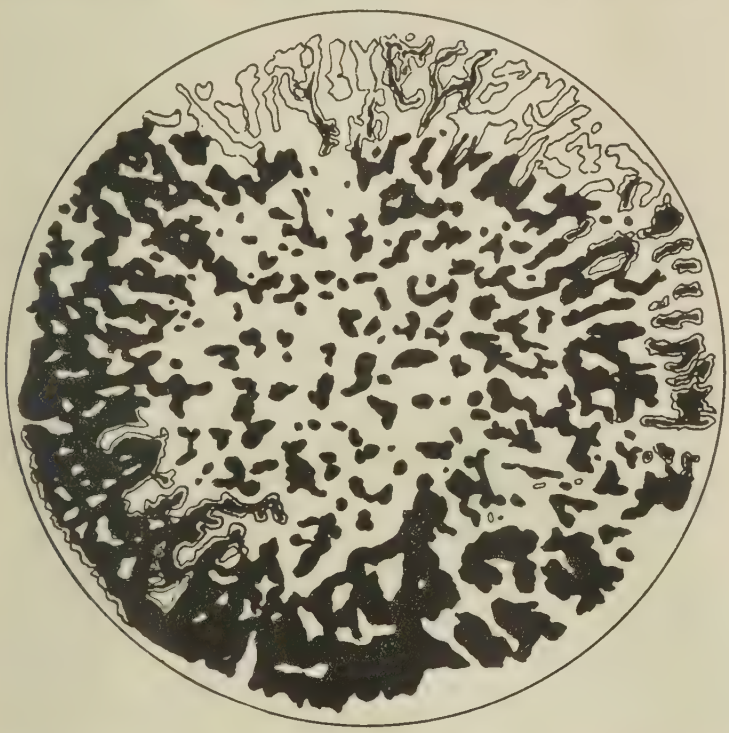
I a. *Cultures producing many sclerotia.* Growth is as fast as in control cultures, but when a culture is two to three days old it is readily distinguished from a control of the same age by the much thinner and lower growth, the formation of a dense sheet of superficial hyphae about the point of infection, and the presence of a relatively small number of weakly branched main hyphae straggling outwards across the medium from the dense centre. Young colonies produce little aerial mycelium, and show a straggling edge of separate main hyphae (Pl. XXXVII, Fig. 12), which contrasts strongly with the dense even margin of normal cultures (Pl. XXXVII, Fig. 10). The medium is rapidly acidified around the colony. When freshly prepared, the medium has a pH of about 7 ; around the edge of a growing colony, a pH value of 3 may soon be demonstrated. If the medium is prepared in such a way that it is slightly turbid with suspended magnesium phosphate, the effect of the acid secreted may be seen in the appearance around the fungus of a clear region a millimetre or so in width, due to the solution of the phosphate. This effect is not specially apparent in cultures of the normal form, which, however, acidifies the medium.

The denser central region extends as the colony grows, and by the time that the edge of the medium is reached a thin central cushion of aerial mycelium is present. A weak marginal ring soon forms, and on it and on the cushion scanty crops of conidiophores appear. This occurs when the culture is nine to ten days old, and at the same time rudiments of sclerotia begin to form in very large numbers, generally in radial streaks. Fusion occurs freely among the rudiments, so that large flat crusts of sclerotia form. This variant is the only one of those investigated which shows a definite reaction to Czapek's medium, for on that medium it forms specially good crops of sclerotia. On both the synthetic media used the crusts may cover 75 per cent. of the surface of the agar.

There is little constancy in the way in which the sclerotia darken. In many cultures darkening began by the appearance on the surface of the sclerotia of small, dark brown areas, which spread and coalesced ; in many others, darkening began at the edges and spread inwards, as in the normal form ; in still others the central parts of the crusts darkened completely, but the edges remained brown and translucent for weeks. Often in the earlier cultures a pink tinge was apparent in the young sclerotia. Usually these darkened completely, but occasionally some sclerotia remained very flat and pink. This usually occurred in vaguely defined sectors, and

conidia taken from above these areas sometimes failed to yield good cultures, while at other times they produced the form next to be described.

I b. *Colonies of low, dense white growth.* Growth is rather slower than in controls, but in early stages the colonies are very like those of I a.



TEXT-FIG. 2. A culture of Variant I a with sectors of pink sclerotia (shown by plain outlines), black sclerotia with pinkish edges (shown by outlines and solid centres), and black sclerotia (solid black). Camera lucida tracing. × 1·15.

The central area is, however, denser, and the straggling edge more obvious, as the hyphae produce large numbers of short lateral branches which arise close together and form conspicuous white groups. As a result of this dense branching the course of the main hyphae may sometimes be traced in cultures months old. Acid is secreted more freely than in I a, so that the acidification of the medium proceeds rapidly, and the solution of magnesium phosphate is well seen. Most of the forms of *Botrytis cinerea* which have been investigated give rise to a plectenchymatous layer on the surface of the medium. In I b this layer is developed strongly, and as the cultures age the plectenchyma thickens, is thrown into folds, and stains yellow below. Investigation of this layer showed the presence in it of a

considerable number of irregularly swollen hyphae tightly crowded together, and suggested that it might be regarded as a kind of imperfectly formed generalized sclerotium, an expression of the strong tendency to form sclerotia which is clearly present in I a. In cultures a month or more old small irregular patches of darkly stained regions are abundant beneath the plectenchyma.

The production of conidiophores is always scanty; they do not arise on a central cushion, nor on a marginal ring of aerial mycelium, but appear in scattered patches on the surface of the culture. It is seldom that well-formed conidiophores are seen; if such occur, they usually form at the edge of a colony which has ceased to extend before it had reached the edge of the dish. More often areas of the surface growth assume a biscuit colour, and conidia are formed on those hyphae in a somewhat casual manner. The conidia do not differ morphologically in any way from those of control cultures.

Sclerotia are rarely formed, and, when they do appear, they are limited in number, seldom exceed a millimetre in diameter, are polished, and densely black. These sclerotia may form around the point of infection, or, if a few conidiophores have been formed at the margin of the colony, they may lie beneath those structures. In one culture rudiments of sclerotia appeared in enormous numbers, but they failed to mature. The formation of conidia and sclerotia by I b has not been found to bear any relation to the age of the culture.

Conidia taken from conidiophores arising above areas bearing pink sclerotia in I a have on many occasions yielded mixed cultures of I a and I b. Mycelial transfers from I b in such cultures have yielded this form only. The examination of conidia has not revealed any differences which can be used to distinguish the conidia of the two variants. Thick plantings of conidia from I a give small, dense colonies resembling I b, but the resemblance is only apparent, for if these colonies have room to grow they give I a. The general results of the investigation of Series I suggest that I a is a somewhat instable form, able to produce pink sclerotia occasionally, and then yielding I b; I b, on the contrary, seems to be a permanent variant. A similar form will be referred to in the description of Series VI and VII.

When transfers of few conidia are made from a good culture of I a, and care is taken to avoid choice of material from areas bearing abnormal sclerotia, this variant can be perpetuated cleanly, and retains in a striking manner its power of forming large crops of sclerotia.

The resemblance of I b to *la toile*, described by Beauverie (3, 4) and by Beauverie and Guillermond, (5) is considerable, but, as it always forms some conidia, and may form sclerotia, it is distinct from that sterile form. Examination of the material has not revealed convincing evidence of the

presence of the modified conidiophores recorded by these authors in their *forme intermédiaire*, from which also I b seems to be distinct in its constant behaviour.

TABLE II.

Analysis of Series I, in Periods of Three Months.

Period.	Class of Culture. I a.					I b.		
	1.	2.	3.	4.	5.	6.	7.	
1928.								
July–September	—	10	2	2	—	—	—	
October–December	2	1	1	2	15	9	11	
1929.								
January–March	—	—	—	1	7	—	3	
April–June	6	—	—	3	16	1	4	
July–September	4	—	—	1	16	20	11	
Totals	12	11	3	9	54	30	29	148

Notes.—1. Cultures showing poor sterile growth.
2. Dense cottony cultures.
3. Cultures of I a form, but with central sclerotial mass.
4. Cultures of I a form, but with some pink sclerotia.
5. Good cultures of I a.
6. Good cultures of I b.
7. Mixed cultures of I a and I b.

Series II.[1]

Some of the earlier cultures of Series IV yielded a few poorly formed sclerotia which remained white. Seven of these were removed and transferred separately to fresh dishes. Six of the cultures agreed in all respects with cultures raised from normal black sclerotia. The seventh produced a colony distinguished by the formation of an exceptionally heavy crop of greyish to brownish aerial mycelium, with a scanty crop of conidia, and a few pinkish sclerotia. Conidial transfers from this culture formed the beginning of Series II.

Early growth is as rapid as in controls, but usually somewhat looser. When a culture is about nine days old a heavy tuft of nearly sterile aerial mycelium arises in the centre; it is at first greyish, but soon becomes a dirty brown, spreads in the free space in the dish, and often fills this so completely that the marginal ring of conidiophores is almost hidden. As the dense centre forms, a yellow stain appears in the medium below it, and by the time that the culture is a month old the medium is heavily stained; in colour the staining ranges from yellow, through brown, to purple and black. The staining is not uniform, but in round patches, which suggest the presence of sclerotia; these, however, have not been found in cultures

[1] See note at end of paper.

of Series II. The formation of appressoria is rather capricious; such as appear are normal. Cultures of this series have been under investigation since October 1928: owing to the great consistency shown, thirteen only have been made. The pronounced staining of the medium is the principal character of the cultures of Series II. Heavy staining has not been observed generally at ordinary temperatures in cultures of *Botrytis*. It may occur in small patches in old control cultures, under the tardily formed tufts of more or less sterile mycelium, and it frequently appears in old cultures of the dense white form of Series I, VI, and VII.

Series III.

The first culture was derived from one of the half sclerotia from Culture A. It started rather weakly, produced a scanty crop of conidia and a few small sclerotia of irregular outline, which took four months to darken. The sister culture was of similar character, and was not further used. Conidial transfers from the first culture gave subcultures which produced an abundance of white, sterile, aerial mycelium, very few conidia, and poor, thin, whitish sclerotia, which remained so for months. These characters were soon lost in succeeding transfers, and, as the investigation has proceeded, the cultures of this series have shown a general drift back to normality. The most general characteristic of the cultures of Series III has been apparent in early stages; the conidia have shown very consistently a weakness in starting, as compared with control cultures. This weakness has ranged from a total inability to germinate to a lag in starting amounting to about twenty-four hours. It has not, however, been possible to establish one definite variant as characteristic of the series, and the cultures fall into the following classes:

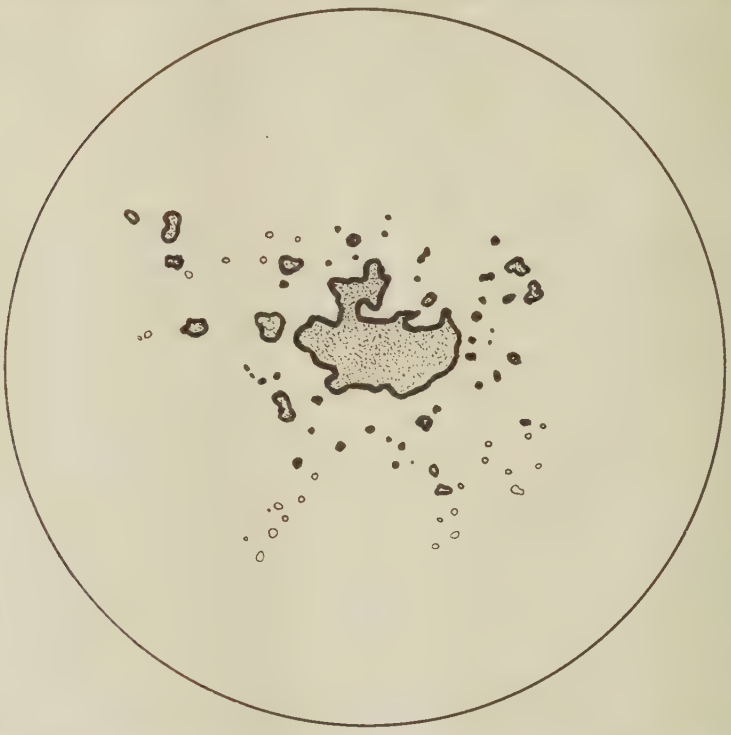
III a. Cultures of very weak growth. Of the 174 cultures which are shown in Table III, 41 (including 34 conidial transfers and 7 mycelial transfers), have failed to develop growth of macroscopic size, and 16 (including 12 conidial transfers and 4 mycelial transfers) have given weak and persistently sterile colonies; in all, microscopic examination showed that the inoculum had been successfully transferred; in many, ungerminated conidia, or conidia showing but faint signs of growth, lay for weeks on the surface of the medium. The inocula which gave these curious results had always been taken from above areas in the parent cultures which bore pink sclerotia, or from a portion of the mycelium which showed specially weak growth. Table III shows that the mere age of the inoculum has nothing to do with the failure to produce a strong colony, and it was often found that sister cultures, started from the same lot of conidia, and treated in exactly the same way, sometimes gave good growth, and sometimes poor or no growth.

After a connexion had become apparent between weak growth and

conidia taken from special areas in the parent cultures, many samples of conidia from such areas were examined, but significant differences in either appearance or size could not be found. In stained material it was seen that the conidia contained on an average about five nuclei, which were too small for satisfactory observation, and differences were not noted. Examination suggested that the hyphae of a weakly growing mycelium were more vacuolate than those of a more active mycelium, but nothing definite could be established. The fact appears to be that if conidia are taken from above pink sclerotia, or if mycelium be taken from regions of feeble growth, such material is likely to give rise to weak cultures; the explanation is still to be found.

III b. Cultures showing a central aggregate. The conidia take about a day longer to germinate than do conidia in control cultures, and in early stages of growth almost all the mycelium lies on or in the medium, and often shows extraordinary curvatures in the main hyphae (Pl. XXXVII, Fig. 11). Ten days may be required to cover a surface which is covered by the normal form in five to six days, and, in the low growth, radiating areas of somewhat greater density soon become apparent. When the culture is about a fortnight old it bears a scanty crop of conidiophores scattered about the surface and a thin central cushion of mostly sterile aerial mycelium. Rudiments of sclerotia appear crowded together, but at first separate from one another, under the central cushion, and others develop in the radiating areas of denser growth. These areas become narrower as they pass outwards, so that a vaguely stellate appearance is suggested, the points of the star reaching about half-way across the dish. The sclerotial rudiments in the centre assume a distinctly pinkish tinge as they develop, and soon fuse into a central aggregate; in this, the components are at first indicated by reddish lines of separation, but as the whole darkens the lines disappear. The larger sclerotia in the radiating areas enlarge, become pinkish, but usually remain separate; those at the ends of the arms enlarge but little, and are persistently pink; those nearer the central mass may ultimately blacken completely (Pl. XXXVII, Fig. 4). One of the early cultures of this sort, when seventy days old, showed a faintly brown, translucent central mass, with scattered brownish sclerotia around it, and small pink ones lying peripherally; the general appearance of the culture is shown in Text-fig. 3. As the work proceeded, this tendency to produce a central aggregate became much weaker; transfers sometimes gave a central aggregate, sometimes yielded cultures with pink sclerotia, but not a central aggregate, and sometimes produced cultures belonging to Class III d. A culture which was made five months after the one just described, by transfer of conidia from a parent showing a central aggregate surrounded by a fair number of black sclerotia, gave signs of the formation of a central aggregate when it was eighteen days old; the rudiments failed

to fuse completely, and many black sclerotia arose subsequently in the peripheral region. The appearance of this culture when seventy days old is shown in Text-fig. 4; there are but vague indications of any radial arrangement, and the old culture, had its history not been known, would

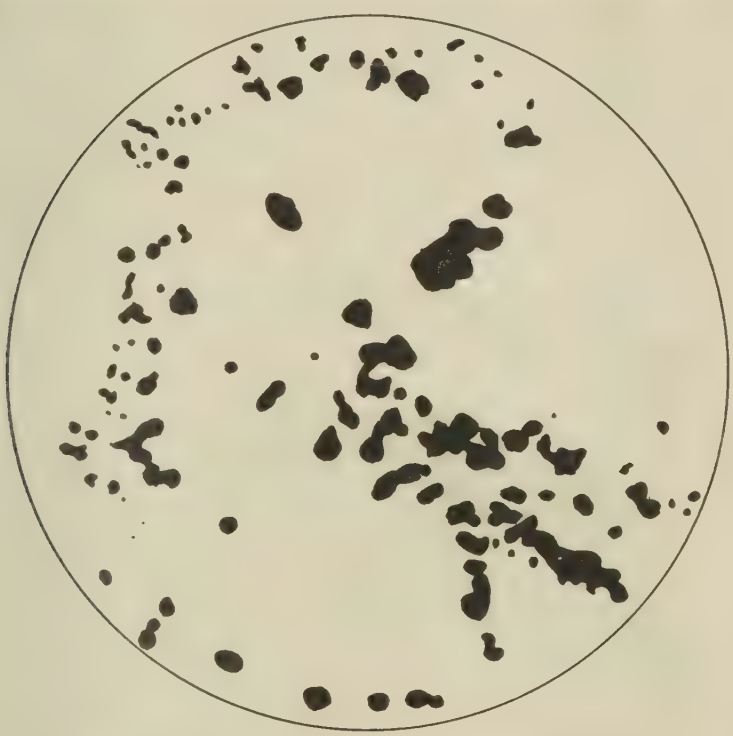


TEXT-FIG. 3. A culture of Variant III b grown in October 1928. The central aggregate is well defined, and the radiating groups are clearly shown. Sclerotia filled in by dots remained brown and translucent; sclerotia shown in outline remained pink. Camera lucida tracing. × 1·15.

not have been readily distinguishable from a culture of Class III d. Cultures showing a central aggregate have not been obtained since July 1929, when the series had been under investigation for a year. It seems likely that the capacity to produce them has been lost as a result of continued subculturing.

III c. Cultures producing pink sclerotia. In early stages these cultures are only distinguished from those of Class III b by slightly stronger growth and less tendency to form curved main hyphae. Conidiophores begin to form after some ten days, and arise in tufts from small dense areas on the surface of the mycelium, or from localized spots in the scanty aerial mycelium; a day or so later rudiments of sclerotia appear on the medium,

about half-way between the centre and the periphery. Some of the rudiments soon assume a definite outline, and blacken completely, requiring maybe a month to complete the process; others, distinguished by a rather vague outline, give rise to thin sclerotia which remain brown or pink for



TEXT-FIG. 4. A culture of Variant III b grown in May 1929. The central aggregate is not well defined, radiation is very vaguely shown, and the sclerotia are black. Camera lucida tracing. × 1·15.

months. These abnormal sclerotia may occur scattered among the dark ones, but they generally form in patches, either close to the edge of the medium or in ill-defined sectors. The latter are areas of weak growth ; in young cultures they may be distinguished by their slower spread and general thinness, and in mature cultures they bear fewer conidiophores than are present on areas carrying black sclerotia. A number of cultures were started from pink sclerotia, in the hope that cultures could be obtained in which such alone developed ; this hope was not fulfilled, for the cultures either resembled the parents or gave cultures belonging to III d.

Inspection of Table IV suggests that the tendency to the production of pink sclerotia is becoming weaker.

III d. Cultures differing from the stock strain in little but general weakness of growth. Young cultures of Class III d are not readily distinguishable from those of Class III c; the growth is slower, and the production of aerial mycelium much weaker than in the corresponding controls. When the margin of the medium is reached a few appressoria may be formed, but such as form are usually light in colour and seldom macroscopic; in the preceding classes appressoria are rarely seen. The formation of conidiophores and of sclerotia proceeds as in III c, but the sclerotia all blacken, and usually do not blacken much more slowly than in control cultures.

TABLE III.

Analysis of Series III, according to the Age of the Parent Culture. July 1928 to August 1929.

Age of parent, days.	Class of resulting Cultures. III a.	III b.	III c.	III d.	III e.	
5–10	14	1	1	—	—	
11–20	14	1	10	13	2	
21–30	7	4	5	11	3	
31–40	6	1	5	2	2	
41–50	9	5	10	13	5	
51–100	3	1	2	6	4	
101–137	4	5	3	1	1	
Totals	57	18	36	46	17	174

TABLE IV.

Analysis of Series III in Periods of Three Months.

Period.	Class of Culture. III a.	III b.	III c.	III d.	III e	
1928.						
July–September	2	—	2	6	—	
October–December	33	7	13	17	8	
1929.						
January–March	12	6	2	3	2	
April–June	4	4	13	5	7	
July–September	6	1	6	15	21	
Totals	57	18	36	46	38	195

Cultures of III d form an important fraction of the series; they have appeared quite erratically among other and more aberrant cultures. Transfers from them have yielded similar cultures, or cultures difficult to distinguish from the normal form.

III e. Nearly normal cultures. These cultures have been obtained when conidia have been transferred from specially dense conidial areas in cultures of III d, or when conidia have been taken from tufts on old sclerotia. In addition, most of the cultures of Series III, which were made

during the hot weather in August and September, 1929, could hardly be distinguished from control cultures. They seem to have reverted completely to the original form.

Series IV.

Series IV began with the transfer of a whole light-coloured sclerotium from Culture A. Growth was at first low and loose, but when the culture was nineteen days old the centre of the dish was occupied by a dense cushion of white cottony mycelium bearing a few conidiophores; growth had stopped before the edge of the medium was reached, and sclerotia did not form. At this time conidia were transferred to fresh media, and from these cultures single colonies were isolated.

These cultures, and those which immediately followed them, produced some pink sclerotia, as well as a dense central cushion of aerial mycelium. From one of these cultures four pink sclerotia were taken. They gave cultures, three of which were the starting-points of Series V, VI, and VII, respectively; the fourth was not further investigated. Conidial transfers from the early cultures of Series IV, taken from above pink sclerotia, failed to yield growth. This is comparable with results obtained in other series. On the other hand, conidial transfers from above black sclerotia gave rise to a series of cultures not differing in any important respect from normal. On the whole, these cultures gave more and larger sclerotia than the corresponding controls; but although the series was carried on for a year striking differences were not obtained.

Series V, VI, and VII.

These series began with cultures made from single pink sclerotia taken from an early culture of Series IV. In all three growth was at first low, with very little aerial mycelium; it was noted that the pink sclerotia soon blackened after they had been transferred to fresh media. The surface of the medium was covered in eight to nine days, then a scanty crop of conidiophores formed, a central cushion of aerial mycelium developed, and rudiments of sclerotia became visible. All three cultures ultimately produced so heavy a crop of cottony aerial mycelium that the free space in the dish was filled, and pitchy black appressoria were formed against the under-side of the lid. Sclerotia appeared in all, and some of these remained pink for months; black sclerotia were also formed, a few of which were surrounded by a purple stained area.

From these cultures conidial transfers were made and single colonies isolated; the further history of these lines will be considered separately.

Series V.

Series V is of special interest. One line of development in the series shows, perhaps even more clearly than in Series III, a gradual return to

normality ; another line has resulted in the isolation of a strain distinguished by the production of microconidia, and of considerable stability.

The cultures of Series V fall into the following groups :

V a. Cultures showing weak growth or no growth. As in Series III these cultures have been obtained by transfers from areas bearing pink sclerotia. They do not need further discussion.

V b. Cultures producing nearly sterile, weak, white aerial mycelium. Some of the conidial transfers from above pink sclerotia gave weakly growing cultures which remained nearly or quite sterile, did not produce appressoria, and never formed good sclerotia. One such culture, when twenty days old, produced fourteen small red spots on the surface of the medium, which looked like sclerotial rudiments. These did not change after further observation, and when transferred to fresh media failed to give growth. Another culture, when eighty-six days old, yielded three bright ruby red sclerotia, which appeared normal in structure. One was examined, and two portions of it placed on fresh media ; the others remained unchanged for five months. Of the transfers, both gave weak growth. A few poor conidial groups were seen when the original culture was 141 days old, and transfers from these gave the microconidial strain.

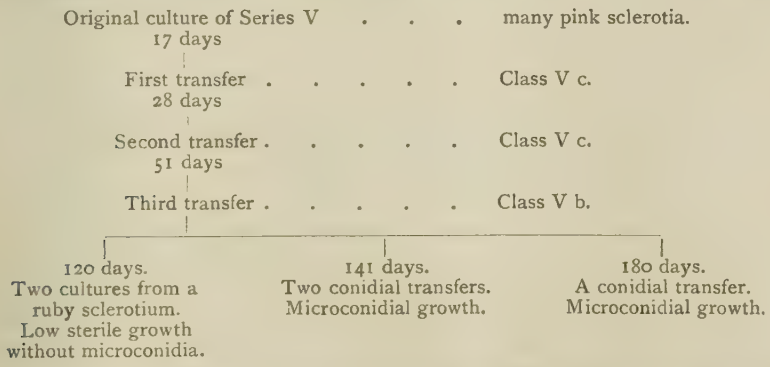
V c. Cultures producing radial streaks of pink sclerotia. One of the sub-cultures from the original culture of the series yielded a slow growing colony, on which conidiophores did not appear until the fourteenth day. The aerial mycelium was distinctly pinkish, and the sclerotial rudiments, which appeared with the first conidiophores, were also pink. The larger sclerotia, which lay towards the centre of the dish, darkened in ten days ; those which lay towards the periphery in radiating lines (Pl. XXXVII, Fig. 5), not ending in a point as in Series III, remained pink. Conidial transfers made when the culture was twenty-eight days old gave rise to two more cultures showing the same phenomena ; transfers from these failed to reproduce the condition ; one gave a culture of class V b, the others gave V d.

V d. Cultures with pink sclerotia. In all, twenty-two cultures of Series V developed some sclerotia which failed to blacken, remaining pink or brown. They appeared most abundantly in the earlier part of the work. Those obtained in the autumn of 1928 were characterized by slow initial growth (one to two days behind the controls), the production of but little aerial mycelium, the presence of a raised edge of pink hyphae at the advancing edge of the mycelium, and the abundant production of pink appressoria on the side of the dish, and of very black appressoria in a strong ring where the lid rested on the side of the dish. The crop of pink appressoria was so strong that the cultures showing this character could be identified as they stood in the stacks. During 1929 the formation of pink appressoria, pink sclerotia and pink aerial mycelium gradually fell off, so that there was gradual merging into cultures of the next class.

V e. Cultures producing a weak crop of conidiophores. Most of the cultures of Series V fall into this class (Pl. XXXVII, Fig. 6). The early cultures began very like those which produced pink sclerotia, and only differed from them in the behaviour of the sclerotia. These were rounded, and definitely pink at first, but they usually blackened completely in about ten days: the aerial mycelium was pinkish, and pink appressoria formed freely. As time went on, the tendency to show a pink tinge in early stages became weaker, so that the later cultures of the class differed from control cultures in slightly slower growth, in a tendency to form rounded rather than radially elongated sclerotia, and in the general production of a thin crop of conidia. In this condition, the cultures may be readily identified on inspection, but it is impossible to state any distinct character which separates them from control cultures. During the hot weather these cultures gave much heavier crops of conidia, with a good deal of brownish aerial mycelium, and could not be distinguished from controls, which at that time also showed a tendency to give pink appressoria. These cultures seem to have reverted completely to the original form; they appear in Table V under V f.

V g. Cultures of dense white character. Three transfers made from cultures of Class V e during hot weather gave colonies like those of I b. This is not altogether surprising, for this variant is the prevailing form in Series VI and VII, which arose from pink sclerotia formed in the same culture as that which gave Series V. As I b yields but a scanty crop of conidia, it does not seem possible that these three cultures can have arisen from contamination; had this been the case it is much more likely that either the stronger normal form or the ubiquitous *Penicillium* would have appeared.

V h. The microconidial strain. The origin of the microconidial strain is as follows:


Original culture of Series V . . . many pink sclerotia.
17 days
First transfer . . . . . Class V c.
28 days
Second transfer . . . . . Class V c.
51 days
Third transfer . . . . . Class V b.
120 days.
Two cultures from a ruby sclerotium.
Low sterile growth without microconidia.
141 days.
Two conidial transfers.
Microconidial growth.
180 days.
A conidial transfer.
Microconidial growth.

It is clear that the microconidial strain has distinctly aberrant cultures in its line of descent.

Once microconidia were obtained difficulty was not experienced in culturing the strain, for they germinate readily.

Allowing for slight individual differences the thirty-nine cultures of V h. may be included under the following general description.

Both mycelial and conidial transfers have been made, the former from young cultures, the latter from older ones. Germination is visible by the second day ; a low and silky colony is formed (Pl. XXXVII. Fig. 7), covering the medium in about ten days. On media 1–2 mm. in thickness, however, growth may cease before the edge is reached ; acid does not appear to be secreted into the medium. A very slight amount of aerial mycelium is formed, and the whole colony is nearly transparent, and appears of the same colour as the medium. Growth is mostly submerged, and branching dense and even. Indications of groups of microconidia are seen when the culture is about three weeks old ; the groups are small, and often invisible to the unaided eye, and so dark green in colour as to appear black to the lens. Frequently, but not always, when a colony has reached a diameter of about 4 cm., further growth takes place from the centre, at a higher level in the medium than that occupied by the earlier growth. This gives a layered effect, often a stellate effect, as the layers may run out in triangular patches. The whole has a filmy appearance, quite distinct from any other of the cultures seen in this investigation.

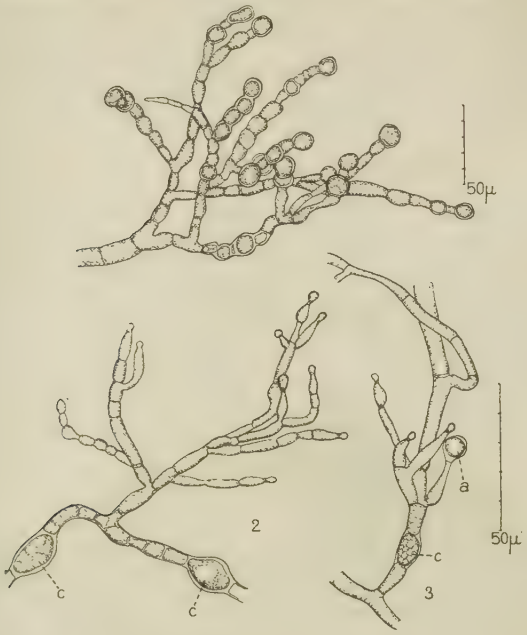
When young the hyphae are of even diameter, but when the culture is a week or so old, the hyphae may be converted into chains of chlamydospores, or into chains in which such spores alternate with unaltered hypha ; this phenomenon was most frequent in early cultures of the form (Text-fig. 5, 1). A complete morphological investigation of this interesting form has not yet been made; indications have been seen that the chlamydospores are not invariably formed by the transformation of existing branches, but often arise on the submerged mycelium from short lateral branches which, in everything but size, recall the sterigmata on which microconidia are formed (text-fig 5, 3 a).

Microconidia are always formed above the surface of the medium. In view of the extensive literature dealing with these structures (Brierley, 6 ; Istvanffi, 10 ; and the papers mentioned by these authors), it is unnecessary to embark on a thorough description.

Sclerotia which have remained creamy in colour and translucent for two months were obtained in one of these cultures (Pl. XXXVII, fig. 8). So far, transfers from this culture have failed to yield sclerotia of any kind. In three cultures in which the normal form and the microconidial form were grown together, the latter gave rise to extraordinary sclerotia—whitish, flat, and presenting the general appearance of a piece of dirty ice. The normal form grew around, but not over these sclerotia.

The microconidial form has retained its general characters in culture

from May to September 1929[1]; the latest cultures show a little more development of aerial mycelium, less inclination to form submerged chlamydospores, and a tendency to produce successive layers of growth in circular patches, and not in a stellate pattern. These changes may indicate



TEXT-FIG. 5. The microconidial strain, Variant V h. 1. Chains of chlamydospores, swollen hyphal segments, and almost unaltered segments. 2 and 3. Young stages in the formation of groups of microconidia. 3 a shows a sterigma which has grown into the medium and formed a chlamydospore. 2 c and 3 c Chlamydospores on hyphae just beneath the surface of the medium.

a return to more normal conditions. Brooks (8), who obtained an apparently similar form on a special medium, records that it soon reverted to normal.

TABLE V.

Analysis of Series V, in Periods of Three Months.

Period.	Class of Culture.								
	V a.	V b.	V c.	V d.	V e.	V f.	V g.	V h.	
1928.									
October–December	12	1	3	12	20	6	—	—	
1929.									
January–March	1	4	—	6	11	3	—	—	
April–June	1	2	—	4	25	1	—	6	
July–September	—	1	—	—	6	34	3	33	
Totals	14	8	3	22	62	44	3	39	195

[1] See note at end of paper.

Series VI and VII.

The origin of these series is given on p. 845. In both series the dense white form corresponding closely with that obtained in Series I has been obtained. Owing to the tardy formation of conidia by this form only fifty-four cultures have been made in the two series, and, whether fresh cultures have been prepared by means of conidial transfers or by means of pieces of mycelium, the form has appeared in all the cultures. As the form was described in Series I, it needs no further treatment here.

Series VIII.

Series VIII needs but brief comment. It began with one of the whole light coloured sclerotia from Culture A, and the first few cultures produced a good deal of whitish and sterile aerial mycelium. Soon, however, transfers began to give cultures distinguished from control cultures only by the production of a few whitish sclerotia (Pl. XXXVII, Fig. 9). Growth from these gave similar cultures, in which the capacity to form white sclerotia was lessened, and after three months of culture this series was allowed to lapse, as nothing but normal cultures appeared.

Conclusion.

The evidence presented in the foregoing pages is sufficient to show that cultures of *Botrytis cinerea*, derived immediately from heated spores, or belonging to a stock originating from heated spores, may present characters distinct from those shown by companion cultures of the normal strain, made at the same time, and grown under the same conditions. During three years of culture, under ordinary laboratory conditions, the normal strain, whether grown from single conidia, mass transfers of conidia, or pieces of hyphae, has not yielded any variant which could be perpetuated in a series of cultures, nor, at an age when the peculiarities were clearly visible in cultures of the variants, have comparable peculiarities been visible in the controls. Although cultures of the normal strain, when started from sclerotia, have yielded subcultures producing heavy crops of sclerotia, it has not been found possible to establish a sclerotial strain in this way, nor to transmit the tendency to cultures inoculated by means of conidia.

In old cultures, and in cultures grown at relatively high temperatures, the normal strain has shown a tendency to produce unusual growths, but these abnormalities could not be transmitted to subcultures : they occurred only under special conditions.

As the normal form has been so constant in its behaviour, it seems unlikely that the variants, which have been isolated and grown side by side

with it, can be explained on the supposition that they are merely normal fluctuations, likely to occur in any series of cultures of *B. cinerea*, grown for a sufficient length of time. Since the variants have been obtained only from cultures with a heated spore in the line of descent, it seems unnecessary to seek for any explanation other than that suggested by the evidence.

The experiments with heated spores have shown the same capriciousness in results noted in the earlier work on *Eurotium herbariorum*. This is in accordance with expectation, for there is no known method of obtaining a standard sample of spores for experimental purposes ; the spore contains several nuclei, and its potentialities are unknown. Even were it possible to subject single spores from the same head of conidia to identical treatment, it by no means follows that all would react in the same way. The biologist cannot hope for such constancy in his results as is obtainable by the chemist, who starts from surer foundations. A further difficulty arises in the selection of a standard with which to compare the results of experiments. The only possible way is to secure a strain of the fungus which appears to possess the general characters of the species, to establish its constancy in culture, under the proposed conditions of experiment, and to use it as an arbitrary standard. The choice of the stock strain used in the present investigation seems to be justified by the constancy it has shown during nearly three years of culture : the forms obtained from it may justifiably be regarded as variants of the standard chosen.

Forms resembling the variants described here, and possibly identical with them, have been isolated from naturally occurring material, or have been produced experimentally by workers with *B. cinerea*. Paul (11) records a number of strains isolated from vegetable substrata, and it is clear from the accounts of other investigators that a variety of forms may be obtained from wild material. A variant characterized by the habitual formation of light coloured sclerotia has been described by Brierley (7), and maintained in culture for years ; it appeared suddenly, and its origin is obscure. Beauverie (3) produced a white sterile mycelial strain of *B. cinerea* by prolonged exposure of the ordinary form to a comparatively low temperature and great humidity on a poor substratum.

It is impossible to explain how distinct strains isolated from rotting plants came into being, as the history of such material must remain unknown ; under natural conditions, however, spores could easily be exposed in sunlight to heating as intense as that used in the experiments described in this paper, and there is no reason to suppose that variants arise only as a result of treatment by heat. The resemblance of artificially induced variants to naturally occurring strains is not surprising : experiment is hardly likely to bring about the appearance of entirely new forms.

A piece of evidence drawn from another organism shows that agree-

ment may exist between abnormalities developed by experiment and abnormalities found in untouched plants. Gain (9) has described the results of exposing the embryos of *Helianthus annuus* to desiccation, followed by severe heating; among the abnormalities noted were some peculiarities in the shape of the leaves. Similar abnormalities were described by the author (1) from an old plant of *H. annuus*, which, so far as is known, was not exposed to unusual conditions. Clearly, in the sunflower, exposure of the embryos to heat is not the only means by which the development of malformed leaves may be induced. The formation of similar abnormalities by plants grown from heated embryos, and by an old plant, may be significant. A parallel exists in another respect between the work of Gain and the present work on *Botrytis*; some of the plants of *Helianthus* flowered, but they did not yield fertile seed. The comparison is not close, but the production of enfeebled conidia by *Botrytis* may be recalled.

Of the variants induced in *Botrytis* four differ notably from the original strain. They are: the dense white form of Series I, VI, and VII, the sclerotial strain of Series I, the variant of Series II, characterized by the free production of brownish aerial mycelium, and the microconidial strain of Series V. The first two are probably permanent, the others possibly so; all have remained constant in culture for months, and all have subcultured cleanly. The remaining variants appear to resemble the transitory variants noted in *Eurotium*; in that fungus they were not subjected to prolonged investigation. When these variants first appeared in *Botrytis* they were sharply distinguished from the normal strain by a tendency to develop a light pink colour in some or all of the young organs, and to yield limited crops of conidia; a few showed unusual grouping of the sclerotia, and all grew weakly in young stages. Many of these peculiarities were indicated in cultures of the stock strain grown at temperatures around 26° C., but from these cultures the special features could not be transmitted to subcultures grown at lower temperatures. The possibility that prolonged culture at a fairly high temperature might yield variant forms has not been tested.

As culture succeeded culture of the transitory variants, their distinguishing features became more weakly expressed; the production of the pink pigment soon diminished; the tendency to weak growth and the development of reduced crops of conidia lasted for more than a year. The general drift back to normal shown by these variants, and analysed in Tables IV and V, is perhaps even more striking evidence that the original changes were due to the disturbing effect of heat on the spores than is the isolation of permanently altered forms. The gradual resumption of the characters of the normal strain, with the slow and orderly disappearance of one distinguishing feature after another, and the accession of strength of growth as the amount of pink colour in the young organs fell off, suggests

that recovery was taking place from the condition of injury set up by the heating of the spore.

The development of a pink pigment in the mycelium, appressoria, and sclerotia of *Botrytis*, appears to be a matter of some interest. The phenomenon has been seen in tufts of aerial mycelium produced by old control cultures of the normal strain, in the aerial mycelium and appressoria of cultures of the normal strain grown at temperatures above those most favourable for development, and in all parts of some of the non-stable variants. A definite connexion undoubtedly exists between the appearance of the pink colour and lack of vigour in the fungus. In the transitory variants most of the sclerotia which remained pink were of indifferent quality, noticeably thin and flat, uneven and vague in outline, and associated with areas of weak mycelial growth; with few exceptions, these sclerotia, in appearance and manner of development, suggested structures which just managed to form and no more. They usually sprouted readily when transferred to fresh media, and always gave rise to colonies in which the presence of a good deal of white, sterile aerial mycelium was a marked character; but a culture started from a pink sclerotium has never given one in which pink sclerotia alone developed. The early growth from pink sclerotia was always weak.

In some of the first cultures of Series V pinkness was visible in the young mycelium; in these cultures a tendency to the arrangement of the sclerotia in radial streaks was often apparent, growth was low and weak, and the crops of poorly defined pink sclerotia were heavy. These cultures were markedly different from the corresponding controls, and in their characters suggested profound disturbance of normal behaviour.

Lack of vigour was also shown, especially in Series III, by the conidia developed over areas bearing pink sclerotia; the conidia often failed to germinate. The weakness in the mycelium of these areas was shown by the poor results often obtained from mycelial transfers. In Series I it was shown that conidia taken from conidiophores above pink sclerotia gave the slowly growing dense white form.

A relationship between the development of pinkness and non-viable spores has been observed in *Syzygites megalocarpus*. In 1928 isolations of this species from material on *Amanita rubescens* yielded weakly growing subcultures: transfers from these grew feebly, and produced a few bright pink sporangia which failed to blacken. The sporangiospores appeared to be normal, except in colour, but they would not germinate.

The development of spores of inferior quality in association with the abnormal appearance of a pink pigment does not seem to have been recorded, but a connexion between pinkness and the slackening of ordinary growth has been noted. Robinson (14) showed in *Pyronema confluens* that formation of reproductive organs from branches of arrested growth is

preceded by the development of a slight pink colour in the aerial branch systems, and Whetzel (15) found in *Sclerotinia Erythroniae* that prolonged culture delayed the appearance of sclerotia, and that these sclerotia, when formed, remained flesh-coloured for a long time. Like the pink sclerotia of *Botrytis*, they often produced a weft of white hyphae.

A connexion appears to exist between the presence of the pink pigment and the formation of microconidia. Incubated cultures of the normal form which developed much pinkish aerial mycelium developed microconidia in about five days. The microconidial strain described on p. 847 arose from a line of distinctly aberrant cultures, originally derived from a pink sclerotium, and distinguished by the abundant formation of pink sclerotia. The mycelium of the microconidial strain appears to be faintly tinged with pink.

The nature of the pink pigment is obscure; it has not been possible to obtain enough material to allow of the extraction of the pigment in a useful amount. The methods of Raper and Wormall (12) were tried on pink sclerotia, on the assumption than tyrosin was involved, but the results were negative.

Under ordinary conditions of culture, the appressoria and sclerotia of the normal strain do not pass through a definite pink stage as they blacken, but the experiments with incubated cultures of the normal strain show that the formation of pink appressoria is easily induced by a moderately high temperature. It is not unlikely that the pink substance is an intermediate product in the formation of the darker pigments of mature organs, and that it is stable enough to accumulate, if, as a result of heating, the ordinary sequence of chemical change is disturbed. That the colour is due to an unstable substance is indicated by the failure to establish a permanent variant with pink organs, and by the gradual disappearance of the colour from the successive cultures of Series III and V. The association which has been shown to exist between the occurrence of the pink pigment and general lack of vigour also points to a disturbance of the normal course of development of the fungus.

A deep green pigment is present in the microconidia, and a similar pigment was noted from time to time, especially in the appressoria of incubated cultures. Microconidia have always germinated readily, and conidia taken from cultures with many green appressoria have not shown unusual behaviour.

In addition to these pigments, which hardly appear during the ordinary growth of the normal strain, the brown pigment characteristic of *Botrytis* needs a short consideration. In *B. cinerea* the ripening of the conidia is accompanied by, or sometimes preceded by, the appearance of a brown pigment in the walls of the aerial mycelium. In the series of cultures made during the investigation all stages have been seen from the

white and sterile aerial mycelium through one which is distinctly brown and bears some conidiophores, and the definite brown condition in the normal sporing form, to the deeply-stained and imperfectly-formed conidiophores sometimes seen in Variant I b. It is probable that the heavily stained hyphae observed in cultures of the normal form at 30° C., and in the non-viable form obtained from singed spores, are further stages in this progression. Apparently the formation of conidia and the development of pigmentation in the walls of the aerial mycelium are both indications of a definite condition of equilibrium; when this is disturbed beyond certain limits one or both of the associated phenomena may fail to appear. It seems too that a relation exists between the formation of conidia and of sclerotia and the staining of the medium. Cultures with heavy crops of one or both of these structures seldom show much staining of the medium. When, however, much sterile aerial mycelium is present, a condition which is usually associated with a poor development of sclerotia, and a limited production of conidia, patches of deep purple or black stain usually underlie the mycelium. This condition is specially well shown by the variant of Series II; in variant I b, which forms few conidia, and seldom forms sclerotia, small blackish patches of stain occur freely beneath old colonies. In all the cultures considered, the conditions of growth have been the same; the differences observed are characters of the variants. The details briefly reviewed here are interesting, as they suggest a possible connexion between the retention of the products of metabolism within the fungus, the development of pigmentation, and the subsequent appearance of the organs of multiplication, though they do not indicate any rigid causal relation between these phenomena. The consideration of the staining effects also indicates that one result of exposing spores to heat may be to alter the permeability of the protoplasm, with noteworthy effects upon the general appearance and development of the cultures.

This work has extended the results obtained with Eurotium, and so confirms the earlier work. Recently, similar experiments have given successful results with *Thamnidium elegans*, and carried the investigation into the Phycomycetes: this will form the subject of a future communication. But although changes have now been obtained in three species by exposing their spores to heat little is yet known of the mechanism of the changes. At present the accumulation of more evidence is held to be of greater importance than any premature attempt at explanation.

Summary.

1. Conidia taken from a strain of *B. cinerea* known to be constant in culture were exposed to high temperatures and transferred to culture media. Of 520 cultures inoculated with heated conidia, 424 failed

to develop growth, 12 gave rise to strongly modified colonies, 64 yielded slight variations, and 20 could not be distinguished from control cultures.

2. The most definite variants arose in cultures showing but few germinations. In three instances a blackened stunted form was obtained, resembling colonies of the normal strain when grown at 30° C.; this form could not be transmitted to subcultures. One culture developed a low whitish growth, not further investigated at the time; a similar form is described as Variant I b. Eight cultures produced some normal sclerotia and a few sclerotia which failed to blacken.

3. From a colony known to have arisen from the only spore which survived heating at 66° C. sclerotia were transferred to fresh media after they had remained light coloured for 105 days. They gave cultures distinguished by a tendency to produce a good deal of loose, white aerial mycelium, few conidia, and few sclerotia. This type of culture was only seen in the early stages of the work; before long, new forms with at least some degree of permanence appeared in subcultures, and were isolated.

4. Three variants have been maintained in culture for thirteen months, having passed through about fifty sets of subcultures. Of these variants one produces a low white mycelium with few conidiophores and rare sclerotia: the second develops a moderate crop of conidia, much nearly sterile aerial mycelium, and no sclerotia: a third forms many sclerotia with few conidia.

5. Two series of unstable variants have been investigated. The early cultures of both were distinguished by rather weak growth, associated with the production of pinkish sclerotia in radiating groups; in one series pink mycelium and pink appressoria were also noted. During thirteen months of culture the abnormal features have slowly disappeared and the normal form has been resumed; the reversion seems to have been hastened by the hot weather in the summer of 1929.

6. From one series, through a line of cultures forming many pink sclerotia, a microconidial strain has been isolated; the microconidia germinate readily, and the strain has retained its characters in culture for nearly six months. One culture of the microconidial strain produced white sclerotia, but a strain characterized by the production of white sclerotia only has not been observed.

7. There is evidence of an association between the occurrence of pink pigmentation in the fungus and weakness of constitution; the pigment has not been investigated, owing to lack of sufficient material.

8. The normal pigmentation of *B. cinerea* is briefly discussed.

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October, 1929.

ADDENDUM.

The foregoing paper was written in the autumn of 1929. Since then, the cultures have been carried on and the position is now as follows:

(*a*) Variants I a, I b, V g, and V h, have retained their characters unchanged.

(*b*) Variant II has reverted to the normal form.

(*c*) The normal form continues steady in behaviour.

September, 1930.

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EXPLANATION OF PLATE XXXVII.

Illustrating Dr. Barnes's paper on Variations in *Botrytis cinerea*, Pers.

The cultures were grown on Reidemeister's medium in Petri dishes 9·2 cm. diameter. Figs. 1, 2, 3, 5, 6, 7, and 8 show the upper surface of the culture; Figs. 4 and 9 were taken through the bottom of the dish.

The microphotographs are from living cultures six days old. In them the diameter of the hyphae is exaggerated by moisture lying on the surface of the medium.

Fig. 1. Colony of the normal strain, showing the even turf of conidiophores.

Fig. 2. Variant I a

Fig. 3. Variant I b. The mycelium is sterile.

Fig. 4. Variant III b. The contraction of the central aggregate has caused two cracks to form in the medium. The outermost sclerotia of the radiating groups remained pink.

Fig. 5. Variant V c. The radiating streaks of pink sclerotia are clearly seen, as well as vague radiating patches of aerial mycelium; this was pink when young.

Fig. 6. Variant V e. Tufts of conidiophores occur on the sclerotia, in addition to the scanty crop of conidiophores on the aerial mycelium.

Fig. 7. Variant V h. Culture of the microconidial strain, six days old.

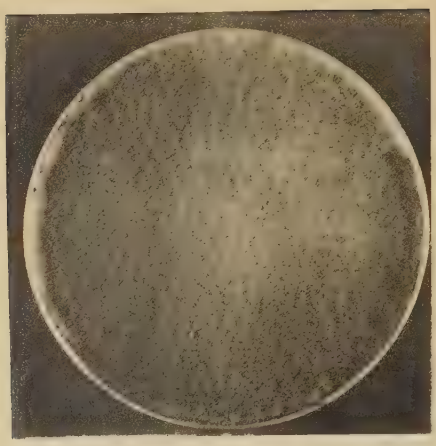
Fig. 8. Variant V h. Culture of the microconidial strain with whitish sclerotia. Material had been removed from the three dark patches.

Fig. 9. The variant of Series VIII. The small white sclerotia remained unchanged from October 1928 to July 1929, when the culture was preserved.

Fig. 10. Portion of the edge of a young normal colony. × 26.

Fig. 11. Portion of the edge of a weakly growing colony of Series III, showing curvature of the hyphae. × 26.

Fig. 12. Portion of the growing edge of Variant I a. × 26. In Variant I b the short lateral branches are more crowded.



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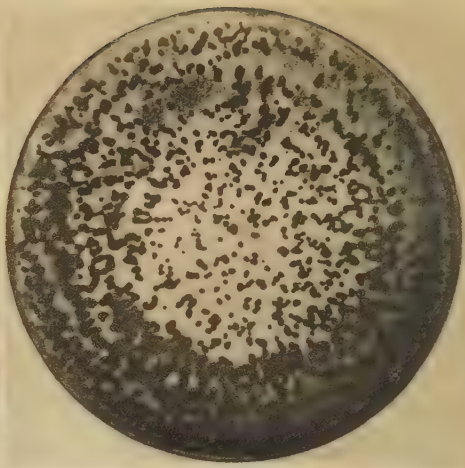

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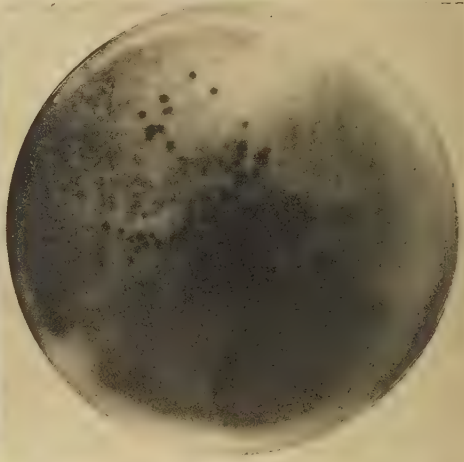

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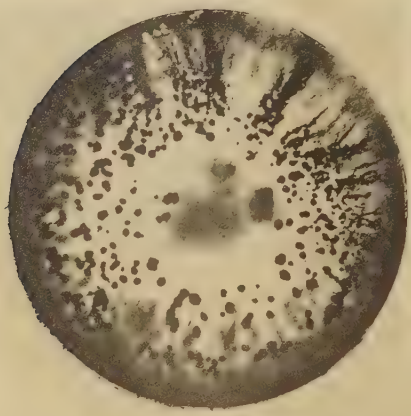

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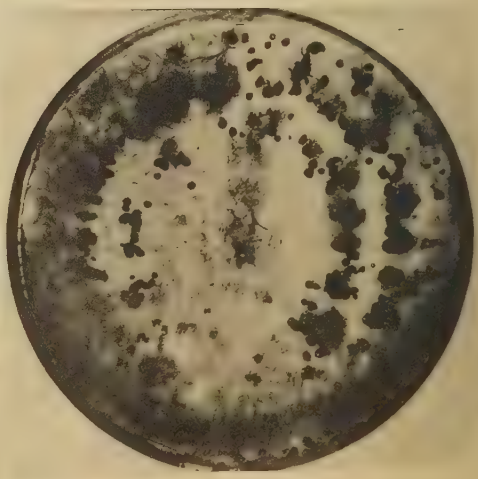

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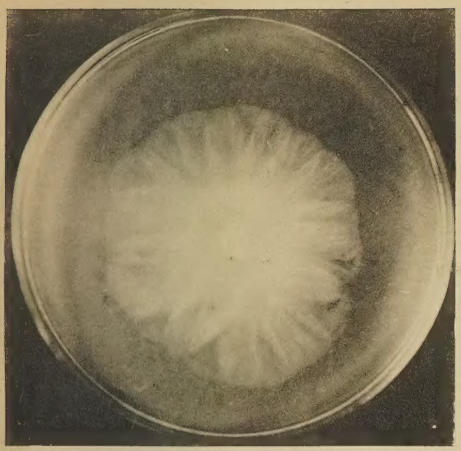

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